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The occurrence of antagonistic bacteria in *Glomus mosseae* pot cultures

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Summary — Biological control of root diseases, carried out by the manipulation of resident microbes or by the introduction of antagonists, should take into account the role played by mycorrhizal fungi in the mycorrhizosphere. In this work we have detected many antagonistic bacteria within the different zones of the mycorrhizosphere of the arbuscular mycorrhizal (AM) fungus *Glomus mosseae*, grown for 17 years in pot cultures. The selected bacteria were actively antagonistic against in vitro mycelial growth of species of the soil-borne pathogens *Fusarium* and *Phytophthora* and on the germination and germ tube growth of *Phytophthora parasitica* zoospores. These results are evidence of the mycorrhizal compatibility of the antagonistic bacteria isolated from the mycorrhizosphere and suggest the possibility of using AM fungi as vehicles of selected microorganisms in biological control of soil-borne pathogens.

Glomus mosseae* / mycorrhizosphere / antagonism / *Phytophthora* / *Fusarium

Résumé — **Présence de bactéries antagonistes dans les cultures de *Glomus mosseae* en pot**. Le contrôle biologique des maladies des racines obtenues par la manipulation de microbes présents autour des racines ou par l'introduction d'antagonistes doit prendre en considération le rôle que les champignons mycorhizogènes peuvent jouer. Dans ce travail, nous avons détecté plusieurs bactéries antagonistes dans différentes zones de la mycorhizosphère du champignon mycorhizogène *Glomus mosseae*, élevé pendant 17 ans en pot. Les bactéries sélectionnées sont des antagonistes très actifs vis-à-vis de la croissance in vitro de champignons pathogènes telluriques tels que des *Fusarium* et des *Phytophthora* et de la germination et la croissance de tubes germinatifs de zoospores de *Phytophthora parasitica*. Ces résultats montrent une compatibilité entre les bactéries antagonistes isolés de la mycorhizosphère et les champignons mycorhizogènes à arbuscules et suggèrent la possibilité d'utiliser ces derniers comme un moyen de propagation de microorganismes sélectionnés pour le contrôle biologique de pathogènes telluriques.

Glomus mosseae* / mycorrhizosphère / antagonisme / *Phytophthora* / *Fusarium

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INTRODUCTION

The association of symbiotic arbuscular mycorrhizal (AM) fungi with plant roots provokes alterations in the physiology and morphology of plants and, as a consequence, quantitative and qualitative changes in root exudates, which largely affect rhizospheric microbes (Meyer and Linderman, 1986; Secilia and Bagyaraj, 1987; Bansal and Mukerji, 1994). In fact, changes in both composition and size of rhizospheric microbial population have been shown to occur in the rhizosphere as a result of mycorrhizal colonization (Bagyaraj and Menge, 1978; Meyer and Linderman, 1986; Azcón-Aguilar and Barea, 1992). Moreover, the extra-radical hyphae of mycorrhizal fungal symbionts are able to explore the soil around the roots and they may, by leaking organic and inorganic materials, provide physical and nutritional substrates for the establishment and metabolic maintenance of microbial microflora (Clough and Sutton, 1978; Tisdall and Oades, 1979). Vancura et al (1989) showed the selective effects of AM extraradical hyphae on the bacteria living in the mycorrhizosphere of *Glomus fasciculatum*. Many of the microbial population shifts occurring in the mycorrhizosphere may involve both bacteria and fungi which might influence the growth and health of plants (Li and Castellano, 1987; Fitter and Garbaye, 1994). Mycorrhiza formation, altering the selective pressure on the population of soil microorganisms, can lead to the selection of antagonistic microorganisms in the mycorrhizosphere: this is one of the mechanisms proposed to explain the role of AM fungi in biological control of plant diseases (Linderman, 1988, 1992, 1994). Sporangium and zoospore production by the root pathogen *Phytophthora cinnamomi* was shown to be reduced in the presence of rhizosphere leachates from AM plants, when compared to leachates from controls (Meyer and Linderman, 1986). In the same way, propagule density of the pathogen *Erwinia carotovora* was reduced in the mycorrhizosphere of mycorrhizal tomato plants compared to control plants (Garcia-Garrido and Ocampo, 1988). Studies by Caron et al (1985, 1986a, b, c) showed that a reduction in *Fusarium oxysporum* f sp *radicis-lycopersici* populations in tomato mycorrhizosphere was associated with a reduction in root rot of mycorrhizal plants compared to non-mycorrhizal ones, and this was attributed to the activity of antagonists. Similarly, larger numbers of actinomycete antagonists towards different fungal and bacterial pathogens were isolated from mycorrhizal than from non-mycorrhizal plants and the numbers varied among plants colonized by different AM fungal species (Secilia and Bagyaraj, 1987).

Works on the biological control of root diseases have been carried out by manipulating resident microbes or by introducing antagonists to reduce the amount of inoculum of pathogens (Baker and Cook, 1974; Papavizas, 1981; Cook and Baker, 1983; Deacon, 1983; Chet, 1987). Nevertheless, these studies have not taken into account the role played by AM fungi in the balance among microbes within the mycorrhizosphere. Our purpose was to select antagonistic microbes actually resident in the mycorrhizosphere, compatible with AM fungi. With this aim in mind, we evaluated the occurrence of antagonistic microorganisms associated with the AM fungus *Glomus mosseae*, grown for 17 years in our pot-culture collection.

MATERIALS AND METHODS

Mycorrhizal fungal culture

The AM fungus used was *Glomus mosseae* (Nicol and Gerd) Gerdemann and Trappe (Kent isolate), kindly provided by Dr B Mosse in 1980. Since then, the fungus was produced and maintained in pot cultures of different host plants (lucerne, strawberry, leek), renewed once a year by collecting sporocarps and mycelium and inoculating them into new pot cultures made up by using sterile sandy soil. The soil contained: 16.4 ppm available P_2O_5 (Olsen), 19.1 ppm available K_2O (ammonium acetate), 0.2% total N (Kjeldahl), 0.56% organic matter, pH (H_2O) 7.3, and was sterilized for 40 min at 121 °C to kill native AM fungi. The pot cultures were maintained in the greenhouse collection of the Institute of Agricultural Microbiology, University of Pisa, Italy (IMA), in individual sun bags (Sigma, Milan, Italy) to avoid external contaminations.

Voucher specimens of the isolate were deposited in the herbarium of the Department of Botanical Science, University of Pisa, *Herbarium Horti Botanici Pisani* (PI) as PI-HMZ 4 and registered in the 'Banque Européenne des Glomales' as a BEG12.

Pathogenic fungal culture

The root pathogenic fungi used were: *Phytophthora parasitica* Dastur (syn *P nicotianae* B de Haan var *parasitica* (Dastur) Waterh) and *Phytophthora cinnamomi* Rands, kindly provided by Dr S Gianinazzi, INRA, Dijon (France); *Fusarium oxysporum* f sp *lycopersici* (Saccardo) Snyder and Hansen, kindly provided by Prof A Zazzerini, University of Perugia (Italy); and *Fusarium*

oxysporum Schlecht f sp *dianthi* (Prill and Del) Snyd and Hans, isolated from carnation-infected plants grown in Sanremo nurseries. *Phytophthora* species were grown on a nutritive agar medium (200 mL•L vegetable juice V8 (Campbell, USA), 2.5 g•L $CaCO_3$, 16 g•L Difco Bacto Agar, pH 7); *Fusarium* species were grown on potato dextrose agar (PDA) medium.

Experimental design

Detection of antagonistic bacteria in the mycorrhizosphere of *Glomus mosseae*

Wet-sieving material from strawberry pot cultures of *G mosseae* was transferred to 100 mL sterile distilled water (SDW) and shaken for 15 min. Antagonistic activity of microbial suspension was evaluated by the cross streak assay method (Filippi et al, 1984; Secilia and Bagyaraj, 1987) using the following pathogenic fungi: *P parasitica*, *P cinnamomi*, *F oxysporum* f sp *lycopersici* and *F oxysporum* f sp *dianthi*. Four days after inoculation, and for the following 5 days, visual assessements of inhibition areas were performed. Each bacterial colony causing growth inhibition was subcultured and tested in in vitro dual cultures for their antagonistic ability against each pathogenic fungus.

Location of antagonistic microorganisms in the mycorrhizosphere

After testing the occurrence of antagonistic bacteria in the mycorrhizosphere of *G mosseae*, this experiment was aimed at locating them in different zones of mycorrhiza influence. Three strawberry pot cultures of *G mosseae* were harvested and mycorrhizosphere analyses were carried out on pot-culture soil, mycorrhizal root surface (rhizoplane) and sporocarp surface.

Mycorrhizal root analysis: The roots were removed from the soil and washed in SDW. The fresh weight root sample (1 g) was put in 100 mL SDW and shaken for 15 min. Serial ten-fold dilutions were then prepared and known aliquots of the suspensions were distributed in five replicate plates.

Sporocarp analysis: One hundred sporocarps of *G mosseae*, collected from the wet-sieved material, were suspended in 100 mL of SDW and shaken for 15 min. Serial ten-fold dilutions were carried out on known aliquots of the suspensions which were distributed in five replicate plates.

The dry weights of mycorrhizal roots and sporocarps were determined by calculating moisture percentage of the fresh material, recovered by filtration on tared weighing dishes, after drying for 48 h at 70 °C and reweighing.

Pot-culture soil analysis: Ten g of fresh weight soil, without root fragments, were sampled from each pot, then transferred to 100 mL SDW, containing 5 g of glass beads, and vigorously shaken for 10 min before serial dilution and plating. Dry weights were obtained after drying an equivalent amount of soil at 80 °C until constant weight. The total number of antagonistic bacteria was estimated by using Waksman agar medium (Bacto casitone 5 g•L [Difco], glucose 10 g•L, NaCl 5 g•L, beef extract 3 g•L, Bacto agar 20 g•L). Then 0.2 mL of each of the suspensions obtained from the serial diluitions described earlier were streaked on the surface of the agar plates, and antagonistic tests on the pathogenic fungi were carried out as described previously.

The plates were incubated at 28 °C for 7 days before quantitative counts. The most representative bacterial colonies were isolated and maintained in Waksman agar, in slant cultures; the selection was based on the following colony characteristics: shape, size, edges, surface and pigment.

Evaluation of antagonistic activity of selected bacteria against *Phytophthora parasitica*

The macroscopically different isolates of antagonistic bacteria isolated from the mycorrhizosphere of *G mosseae* (experiment described earlier), were cultured on Waksman agar spread plates, in the dark, at 24 °C. After 5 days, bacterial colonies were removed from the surface of the agar medium and a sterile Millipore membrane (0.45 µm pore size) was put on the surface of each plate as a physical barrier between the residual bacterial cells and the pathogen. In the controls, Millipore membranes were put on uninoculated Waksman agar plates. Zoospore production was obtained by growing *P parasitica* on V8 agar medium, in the dark, at 24 °C. After 7–10 days growth, the agar was cut into 1 cm squares and put into petri dishes containing 10 mL of SDW. One week later, the petri dishes were chilled at 4 °C for 1 h, then incubated for 30 min at 24 °C, and the zoospore number was estimated. One mL of *P parasitica* zoospore suspension containing about 1 000 zoospores/mL was put on each millipore membrane and plates were incubated in the dark, at 24 °C. After 6 and 12 h, membranes were gently removed from the agar plates and stained with 0.01% trypan blue in lactic acid. Six replications were carried out for each bacterial isolate. The percentage of germinated zoospores and hyphal length of their germ tubes were detected and analyzed by χ^2 test and ANOVA test, respectively. Heat resistance of the putative antagonistic substances produced by the selected bacteria was tested by autoclaving at 121 °C for 20 min the agar medium (spread plates) where antagonistic microbes were grown, pouring it into new plates and inoculating the pathogen as described earlier.

RESULTS

Detection of antagonistic bacteria in the mycorrhizosphere of Glomus mosseae

A high number of microbes causing growth inhibition of any of the four different pathogens tested

was detected in the wet sieving from strawberry pot cultures of *G mosseae*. Thirteen macroscopically different isolates of antagonistic microbes were tested in in vitro dual culture with the pathogens (fig 1). Large differences in the antagonistic ability of the microorganisms isolated were observed, depending on pathogen species, as shown in table I.

Location of antagonistic microorganisms in the mycorrhizosphere

The results of quantitative microbiological analyses, carried out on pot-culture soil, mycorrhizal root surface and sporocarp surface, are reported in figure 2. The total number of antagonistic microbes ranged from 1.6 x 10^4 in pot-culture soil to 6.3 x 10^5 in mycorrhizal roots to 2.5 x 10^5 in sporocarps of *G mosseae* grown in strawberry pot-cultures. These values represent about 1 x 10^{-4}, 1 x 10^{-4} and 1 x 10^{-5} % of the total number of bacteria in pot-culture soil, mycorrhizal roots and sporocarps respectively.

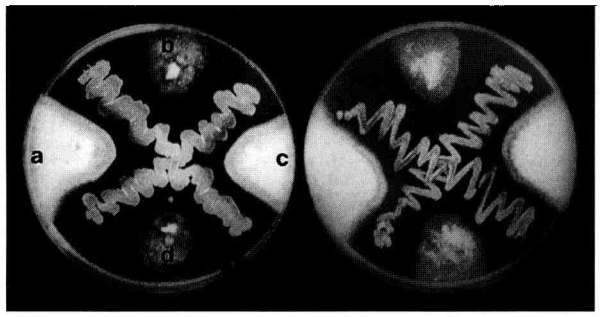

Fig 1. Antagonistic tests in in vitro dual cultures, carried out on BM8 and BM9 isolates (see table I) from *Glomus mosseae* mycorrhizosphere against *Fusarium oxysporum* f sp *lycopersici* (a), *Phytophthora parasitica* (b), *Fusarium oxysporum* f sp *dianthi* (c) and *Phytophthora cinnamomi* (d).

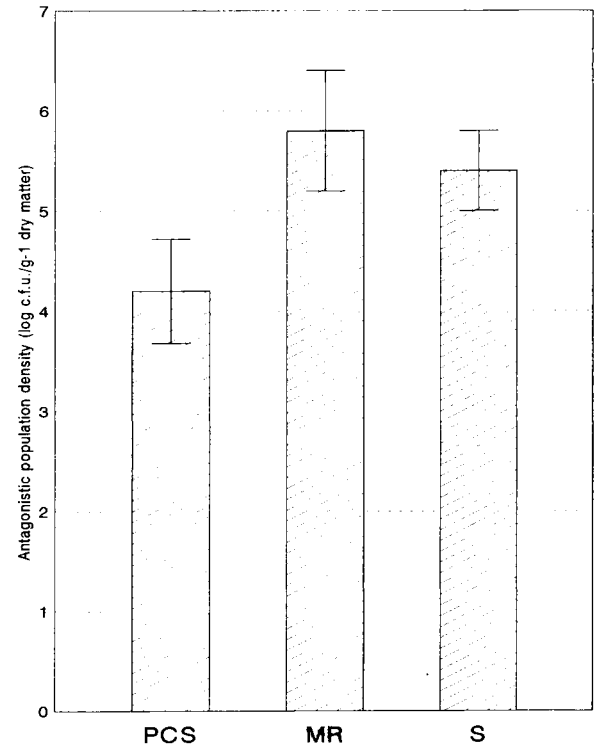

Fig 2. Total number of antagonistic microorganisms in pot-culture soil (PCS), mycorrhizal roots (MR) and sporocarps (S) of *Glomus mosseae*. Vertical bars represent the standard error of the means.

Table I. Microbes occurring in the mycorrhizosphere of *Glomus mosseae* showing antagonistic activity against *Fusarium oxysporum* f sp *lycopersici, Fusarium oxysporum* f sp *dianthi, Phytophthora parasitica* and *Phytophthora cinnamomi.*

	F o lycopersici	*F o dianthi*	*P parasitica*	*P cinnamomi*
B1	–	–	–	+/–
B3	+/–	–	–	+ +
B9	+/–	–	–	–
BM1	–	–	+	+ +
BM2	–	–	–	+/–
BM3	–	–	–	+/–
BM4	–	–	+	+ +
BM6	+	+	–	+ +
BM7	+/–	+/–	+ +	+ +
BM8	+/–	+/–	+/–	+
BM9	+/–	+/–	+	+ +
BM11	+	+	–	+ +
F6	+/–	+/–	–	+/–

– Indicates no hyphal growth inhibition; +/–, +, + + indicate inhibition radii ≤ 0.5, 0.5 – 1 and ≥ 1 cm respectively.

Fourteen macroscopically different isolates of antagonistic bacteria were detected from the different zones of the mycorrhizosphere of *G mosseae*, and subcultured before testing their antagonistic activity against *P parasitica* zoospores.

Evaluation of antagonistic activity of selected bacteria against Phytophthora parasitica

Antagonistic bacteria isolated from pot-culture soil, mycorrhizal roots and sporocarps of strawberry pot-cultures of *G mosseae* were tested for their antagonistic ability to inhibit zoospore germination and germ tube growth of the plant pathogen *P parasitica*. All the microorganisms isolated from the sporocarps and the majority of those isolated from pot-culture soil and mycorrhizal roots significantly reduced the percentage of zoospore germination compared to the control, both 6 and 12 h after zoospore inoculation (table II). Moreover, the occurrence of antagonistic bacteria strongly reduced the hyphal length of germinated zoospores (table II). In contrast, strain H5, isolated from pot-culture soil, though able to hinder mycelial growth of *P parasitica* at 12 h from inoculation, did not show any significant effect on zoospore germination.

Heat resistance of metabolites produced by the mycorrhizospheric microoorganisms showing antagonistic ability was tested. Four bacterial isolates, one from sporocarps, one from mycorrhizal roots and two from pot-culture soil, significantly reduced the germination of *P parasitica* zoospores, even after autoclaving, showing that the putative antimycotic metabolites were heat resistant (table II).

Table II. Effect of antagonistic microorganisms isolated from *Glomus mosseae* mycorrhizosphere on the germination and the hyphal growth of *Phytophthora parasitica* zoospores.

	Zoospore germination (%)		*Hyphal length (mm)*		*Heat resistance*
	6 h	*12 h*	*6 h*	*12 h*	
Pot-culture soil					
H1	9.8 *	19.1 *	20.3 *	39.2 *	+
H4	21.0 *	36.8 *	22.3 *	52.0 *	+
H5	59.6	73.1	45.0	103.7 *	–
Mycorrhizal root					
MR1	30.4 *	20.7 *	38.9 *	44.3 *	–
MR3	34.4 *	55.7 *	82.5	96.1 *	–
MR4	67.1	nd	63.9	nd	nd
MR5	69.5	58.6 *	36.7 *	81.8 *	+
MR7	34.5 *	60.9 *	30.6 *	101.5 *	–
Sporocaps					
S1	37.5 *	42.8 *	46.2	91.9 *	–
S2	34.1 *	37.9 *	37.5 *	36.8 *	–
S3	30.5 *	63.0 *	39.4 *	64.8 *	–
S4	22.0 *	39.3 *	37.2 *	88.9 *	+
S5	30.9 *	69.6 *	18.6 *	100.9 *	–
S6	33.8 *	53.2 *	36.1 *	99.7 *	–
Control	69.7	86.7	56.1	202.4	

* Values statistically different from the control ($P = 0.05$); nd: not determined.

DISCUSSION

The results of this work show that: i) many antagonistic bacteria occur within the different zones of the mycorrhizosphere of *G mosseae* strawberry pot-cultures; ii) selected bacterial isolates are actively antagonistic against in vitro mycelial growth of species of the soil-borne pathogens *Fusarium* and *Phytophthora*; iii) their antagonistic activity can also operate through a direct action on the germination and growth of *P parasitica* infective propagules.

The mycorrhizal fungus, *G mosseae,* used in this work, had been growing continously for 17 years in the IMA pot-culture collection, where it was maintained in sterilized sandy soil and in controlled environmental conditions. Notwithstanding, high numbers of antagonistic microbes were detected not only on mycorrhizal roots, but also on *G mosseae* sporocarps and in the pot-culture soil around the mycorrhizal roots. These findings confirm previous reports on the occurrence of antagonistic bacteria in the mycorrhizosphere (Stack and Sinclair, 1975; Secilia and Bagyaraj, 1987; Catskà, 1994), and suggest that *G mosseae* sporocarps, the only source of microorganisms in our system, harbour compatible bacterial antagonists. The isolation of antagonistic bacteria from the mycorrhizosphere, where their target pathogens live, might ensure their suitability as biological control inoculants, since they belong to the indigenous population of microbes established into the mycorrhizosphere itself (Carrol et al, 1995). The mycorrhizal compatibility should be regarded as a fundamental prerequisite in any biological control project involving the management of microbes antagonistic against soil-borne pathogens.

Our work shows that the antagonistic bacteria isolated from *G mosseae* mycorrhizosphere exert their antagonistic activity, not only on pathogen mycelial growth, but also on the germination and germ tube growth of *P parasitica* zoospores.

Previous works have reported the reduction of sporangium and zoospore production by the root pathogen *Phytophthora cinnamomi* in the presence of rhizosphere leachates from AM plants (Meyer and Linderman, 1986). Accordingly, other authors showed the adverse influence of mycorrhizospheric soil on the population of *Fusarium oxysporum* f sp *radicis-lycopersici* (Caron et al, 1985, 1986a, b, c).

Nevertheless, it has been reported that such studies may show great variability in relation to each plant host-pathogen-AM fungal species combination. In fact, Secilia and Bagyaraj (1987) observed large numbers of actinomycetes antagonistic against the pathogens *Fusarium solani* and *Pseudomonas solanacearum* in pot-cultures of *G fasciculatum* and *Acaulospora laevis*, whereas *Gigaspora margarita* pot cultures harboured many actinomycetes antagonistic against the phytopathogenic bacterium *Xanthomonas campestris* pv *vignicola*.

Future research should be aimed at selecting the best AM fungal strains harbouring efficient microbes antagonistic against specific root pathogens. This is particularly important in view of the management strategy of AM fungi as vehicles for introducing selected microorganisms into peculiar agricultural systems.

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